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MECHANISM OF RESISTANCE OF 'NEISSERIA GONORRHOEAE' TO ANTIBIOTI--ETC(U)
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FINAL REPORT

PERIOD: MAY 1, 1974 - APRIL 30, 1975

PRINCIPAL INVESTIGATOR: ARTHUR K. SAZ, PH.D.

SECURITY CLASSIFICATION OF THIS PAGE (When Data Entered)

REPORT DOCUMENTATION PAGE

READ INSTRUCTIONS BEFORE COMPLETING FORM

1. REPORT NUMBER

2. GOVT ACCESSION NO.

3. RECIPIENT'S CATALOG NUMBER

4. TITLE (and Subtitle)

Mechanism of Resistance of Neisseria gonorrhoeae to Antibiotics

5. TYPE OF REPORT & PERIOD COVERED

Final rept. 1 May 73 - 31 May 1, 1973 - July 31, 1975

6. PERFORMING ORG. REPORT NUMBER

7. AUTHOR(s)

Arthur K. Saz

8. CONTRACT OR GRANT NUMBER(s)

DADA17-73-C-3103

9. PERFORMING ORGANIZATION NAME AND ADDRESS

Georgetown University, School Medicine & Dentistry
3900 Reservoir Rd., N.W.
Washington, D.C. 20007

10. PROGRAM ELEMENT, PROJECT, TASK AREA & WORK UNIT NUMBERS

61102 A, 3A 161102 B71Q
02 B 71 Q 00059

11. CONTROLLING OFFICE NAME AND ADDRESS

U.S. Army Medical Research & Development Command
Washington, D.C. 20314

12. REPORT DATE

November 24, 1976

13. NUMBER OF PAGES

3

14. MONITORING AGENCY NAME & ADDRESS(if different from Controlling Office)

15. SECURITY CLASS. (of this report)

Unclassified

15a. DECLASSIFICATION/DOWNGRADING SCHEDULE

16. DISTRIBUTION STATEMENT (of this Report)

Approved for release; distribution unlimited

17. DISTRIBUTION STATEMENT (of the abstract entered in Block 20, if different from Report)

18. SUPPLEMENTARY NOTES

To be published in Antimicrobial Agents & Chemotherapy

19. KEY WORDS (Continue on reverse side if necessary and identify by block number)

Neisseria gonorrhoeae, ANtibiotic Resistance, Resistance to Penicillin

20. ABSTRACT (Continue on reverse side if necessary and identify by block number)

It was found during the contract term that an inverse relationship existed between the amount of ^{14}C-penicillin G bound and the sensitivity of Neisseria gonorrhoeae to the antibiotic, i.e., sensitive organisms bound more benzyl penicillin than did resistant organisms. Fractionation of the whole cells indicated that binding occurred at the level of the cytoplasmic membrane and again, the inverse relationship occurred. Specificity of binding was shown by pretreatment of preparations with either unlabeled penicillin or cephalothin, in which instances binding was either strongly inhibited or abolished. From 4-9

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SECURITY CLASSIFICATION OF THIS PAGE (When Data Entered)

20. (Continued)

different proteins as determined by gel electrophoresis and/or column chromatography bound proteins.

In sum, resistance to penicillin of the gonococcus can be explained by change(s) in the binding proteins of sensitive cells when compared with resistant cells. When the contract ended, we were just starting to characterize the binding proteins.

Penicillin-Binding to Cell Wall and Cell-Membrane Components of the Gonococcus.

In the previous progress report, I have described experiments indicating that the gonococcus binds penicillin in amounts inversely proportional to the MIC of the antibiotic, i.e. resistant cells bind less than sensitive cells. We have followed this up in the last several months by fractionating the cells and determining the locus of binding.

Cells were collected as described, concentrated and disrupted in Gey's buffer containing crystalline RNA'se and DNA'se in the Braun Tissue Homogenizer for 3 minutes in 30 second bursts with 30 seconds cooling between bursts. The temperature was maintained at 5-10°C by cooling with CO_2. The broken cell suspension (97-99% disruption as observed under phase microscopy) was made 2mM with respect to Mg^{++} by addition of solid $MgCl_2$.

A. Cell Wall Preparation

The broken cell suspension was centrifuged at 2750 RPM for 10 minutes in the cold to remove whole cells and the pellet was discarded. The supernatant fluid containing crude walls and membranes and cytoplasmic constituents was then centrifuged at 25000xg for 15 minutes. The pellet was suspended in an appropriate volume of Gey's buffer and washed three times.

B1 ^{14}C-Penicillin Binding to Cell Wall Preparation

The pellet containing partially purified cell walls was suspended in ^{14}C-penicillin (4 µg/ml). The remainder was incubated at 30°C with shaking for 20 minutes. Binding with 1.0 ml samples was terminated in all instances by the addition of 0.01 ml of unlabeled penicillin G (40 mg/ml) followed by the addition of 10^5 units of Bacto penicillinase. The suspensions were centrifuged at 25000xg a nd washed three times in Gey's buffer. Each pellet finally was suspended in 0.5 ml of buffer and 0.1 ml was counted in a Beckman LS 150 scintillation counter.

Table I shows the results obtained while using 4 µg/ml of ^{14}C penicillin with a specific activity of 1.5×10^5 dpm/µg.

TABLE 1

Binding of ^{14}C-Penicillin to Partially Purified Cell Walls

Strain	MIC	nanomole ^{14}C penicillin bound/gram protein
F18	0.5 µg	48
F19	0.1 µg	4.05
Sensitive	.008 µg	5.8
37492	.008 µg	15.0
62162	0.25 µg	5.0
96120	12.08 µg	3.0

The results are considered insignificant in view of the fact that EM observation of the wall preparations indicated contamination with cell membrane, which as indicated below binds very much higher levels of the antibiotic. The results in Table I further have not been corrected for membrane binding by use of boiled preparations. To determine relative non-specific binding, these results are gross nonomoles bound/gram of protein.

B2. A pellet containing the partially purified cell walls was next exposed with intermittent shaking at 30°C to ^{14}C penicillin at 20 µg/ml for 20 minutes. A parallel aliquot using cell walls boiled for 5 minutes was treated similarly. The results are depicted in Table 2.

Table 2

Binding of ^{14}C-Penicillin to Partially Purified Cell Walls

Strain	MIC(µg/ml)	Nanomole ^{14}C Penicillin bound/gram protein	Nanomole ^{14}C bound/ gram boiled protein
1) 37492	0.008	27	not done
2) 62168	2.0	10	8.3
3) 62162	0.25	11	not done
4) wild section	0.008	37	32
5) 33684	1.0	7.7	2.9
6) F18	0.5	21	26
7) 16577	0.05	16	15
8) 43137	0.125	13	24
9) GC	12.0	11.2	not done

Once more the limited amount of gross binding even with contamination by cell membrane points to the lack of importance of the cell wall's role as a penicillin binding element. If we introduce the boiled specimen correction, then the amount of binding decreases even further. Boiling of the cell wall preparation resulted in a gross amount of clumping which was rather resistant to dispersion and this may account for the higher binding under the boiled protein section seen in #6 and #8.

C1. Penicillin Binding to Cytoplasmic Membrane

Cytoplasmic membranes were prepared from the 25,000xg supernatant by centrifuging at 100,000xg in the cold in a Beckman ultracentrifuge in a.40S rotor. The resultant pellet was suspended in 1. ml of Gey's buffer and exposed to ^{14}C-penicillin at 4 µg/ml for 20 minutes at 30°C. To terminate the binding as with exposure of cell wall, 400 µg of cold penicillin was added, followed after 5 minutes by 10^5 units of Bacto-penicillinase. The suspension was then centrifuged at 100,000xg for 40 minutes, and washed three times with 1. ml of Gey's buffer (after the third wash, the supernatant contained no significant amounts of radioactivity above background). After washing, the pellet was resuspended in 0.5 ml of buffer and 0.1 ml of buffer and 0.1 ml of this was used for counting. As a control an aliquot of the same membrane preparation was placed in a boiling water bath for 2 minutes immediately prior to the addition of the labeled penicillin and this boiled sample was then treated in the same manner as the unboiled. The results presented

in Table 3 have had the boiled membrane values subtracted from the non-boiled membrane (so-called "enzyme protein", following the custom employed by others studying membrane proteins).

Table 3

Binding of ^{14}C-Labeled Penicillin by Cytoplasmic Membrane Proteins

Strain	MIC-μg/ml	Nanomoles ^{14}C Bound/gm. "enzyme protein"
1) 37492	0.008	72
2) 62162	0.25	17
3) F18	0.5	28

C2. Cytoplasmic membranes from 8 strains of N. gonorrhoeae were treated as in C, with one exception. This time a concentration of 20 μg/ml of ^{14}C penicillin with a specific activity of 3.5×10^5 dpm/μg was used. The results are reported in Table 4 as nanomoles of ^{14}C penicillin bound per gram of enzyme protein.

Table 4

Binding of ^{14}C-Penicillin[a] to Membrane Proteins of Various N. gonorrhoeae Strains

Strain	MIC	Nanomoles ^{14}C Bound/gm "enzyme protein" [b]
1) 37492	.008	266
2) EB16577	.05	132
3) GCW	.008	135
4) 43137	.125	17
5) F18	.5	44
6) 62162	.25	32
7) 62168	2.0	18
8) GC20c	12.0	15

a. Penicillin at 20 μg/ml.
b. The boiled "enzyme protein" binding has been subtracted.
c. Isogenic mutant derived from GCW.

When section C is closely examined, several points of interest become evident. There is a marked difference between sensitive and insensitive strains in the binding of ^{14}C penicillin by cell membranes. The overall increase in binding of ^{14}C penicillin by cell membranes on exposure to higher concentrations of penicillin, a phenomenon that has been observed by others, served to amplify even further the differences between resistant and sensitive strains. The actual explanation of the behavior is not known and I am loathe to ascribe it to non-specific binding. A comparison of the data in Table 3 shows that if specimens 1, 2, 3 are compared respectively to 1, 5, 6 in Table 4, the differences between the 3 strains is maintained whether exposed to 4 μg or 20 μg/ml penicillin on increasing the ^{14}C penicillin concentration.

Membranes derived from ^{14}C penicillin sensitive cells bound between 130 and 266 nanomoles of ^{14}C penicillin per gram of "enzyme protein" while the relatively insensitive strains bound between 15 and 44 nanomoles per gram of "enzyme protein".

C3. When two strains, a sensitive (0.008 µg/ml) and a resistant (2 µg/ml) were exposed in parallel to their respective MIC of ^{14}C penicillin, the gross nanomoles of ^{14}C penicillin bound yielded 26 for the sensitive and 5.3 for the resistant strain at MIC.

D. Effect of Cephaloridine and "Cold" Penicillin on Binding of ^{14}C-Penicillin.

To determine whether the binding of the labeled antibiotic was specific, cephalothin (2 µg/ml) or the unlabeled penicillin (2 µg/ml) were added to a membrane binding system for 10 minutes prior to the addition of ^{14}C-penicillin for 10 minutes.

Table 5

Effect of Cephalothin and Unlabeled Penicillin on the Binding of Labeled Penicillin

Strain	43137	37492	718	62168	16577	62162
MIC	0.125	0.008	0.5	2.0	0.05	.25
CM	58	354	83	57	212	38
BCM	41	88	39	33	80	6
Cephalothin[a]	55	184	69	15.3	169	44
Cold penicillin[b]	37	170	51	27	42	11

Preparations CM and BCM were exposed to 20 µg/ml ^{14}C penicillin for 20 min. as previously described in Section C2.

CM = cytoplasmic membrane
BCM = boiled cytoplasmic membrane

a gross nanomoles ^{14}C penicillin binding after pretreatment 2 µg cephalothin/ml for 10 minutes and then treated with 20 µg/ml ^{14}C penicillin for 10 minutes. See Section D.

b ibid a except unlabeled penicillin used for pre-treatment in lieu of cephalothin. See Section D.

Pretreatment with cold penicillin resulted in complete loss of binding in 50% of the strains tested and in a reduction fluctuating between 15% and 30% of the total in the other half. The addition of cephalothin on the other hand caused binding reductions anywhere between 100% and 12% of the total (see table 5). All these figures were arrived at using nanomoles/gram enzyme protein.

E1. Preliminary Work on Separation of Membrane Proteins.

SDS-polyacrylamide disc gel (7.5%) electrophoresis was done on membrane samples using the Weber-Osborn procedure with modification. 100 µl of sample, 70 µl sample buffer and 30 µl of 60% bromphenol blue-containing sucrose were applied per tube and electrophoresed at 4 ma for six hours. The stained and frozen gels were cut into 1.2 mm sections, incubated overnight in H_2O_2 at 56°C and then counted for 20 minutes after 6 hours in scintillation fluid. The results are shown in Fig. 1. Five major bands, which were essentially abolished by pre-treatment of the membrane fraction with unlabeled antibiotic (either penicillin or cephaloridine) are evident. In the case of cephaloridine, there are some binding sites for penicillin which are not abolished by pre-treatment. The strain used in the experiments in Fig. 1 was 37492, MIC for benzyl penicillin = 0.008 mg/ml.

E2. Since there was indication that the disc gel technique was not separating proteins adequately for our needs, we next attempted separation by the slab gel technique as described by Studier (J. Mol. Biol. 79, 237, 1973). The buffer system and polyacrylamide concentrations remained as above and 0.05 ml of membrane preparation was applied to each slot. The gels were stained by Coomasie Blue and destained by 7.5% acetic acid in 5% methanol. The slabs were dried under vacuum in a boiling water bath as described by Maizel (Meth. Virol. 5, 179, 1971).

As Fig. 2 indicates, using purified membranes from strain F19 (MIC = 1.0 µg/ml), 9 clearly defined protein bands appeared, 6 of which migrated faster than Fraction V Bovine Serum Albumin, indicating M.W. of less than 69,000.

E3. We have modified our electrophoresis system with still better resolution of the bands. Currently, the method in use is a discontinuous Tris-SDS buffer with an upper stacking gel of 6% polyacrylamide and a lower separating gel of 7 1/2%. The current was increased to 12 ma until the tracking dye entered the separating gel at which point the current was increased to 25 ma for the remainder of the run. Drying and then radioautography were performed on the bands derived from membranes (F19) exposed to ^{14}C labeled penicillin. Fig. 3 indicates that 14 protein bonds appeared in this system, compared to 5 in disc gel and 9 in the earlier slab method. In the current method of the 14 bonds, 8 exhibited radioactivity (Fig. 3).

Exposure of cytoplasmic membranes derived from sensitive and resistant strains to ^{14}C penicillin and subsequent electrophoresis and autoradiography are further depicted in figures 4, 5 and 6. In contrast to earlier work on F18, only 11 bands appear and of these the ^{14}C binding bands range in number from 5 in F18 (figure 4) to 7 in 1655 (figure 6). In 33684 (figure 5), 6 bands bound ^{14}C penicillin. The intensity of the bands decreases or is abolished on pretreatment with either cold penicillin or cephalothin. In fact in 1655 (MIC 0.05), all but one of the radioactive bands are abolished after pretreatment with both cephalothin and cold penicillin. In all SDS systems, 7 to 8 bands migrate faster than Bovine S. Albumin fraction V which suggests molecular weights of less than 69,000.

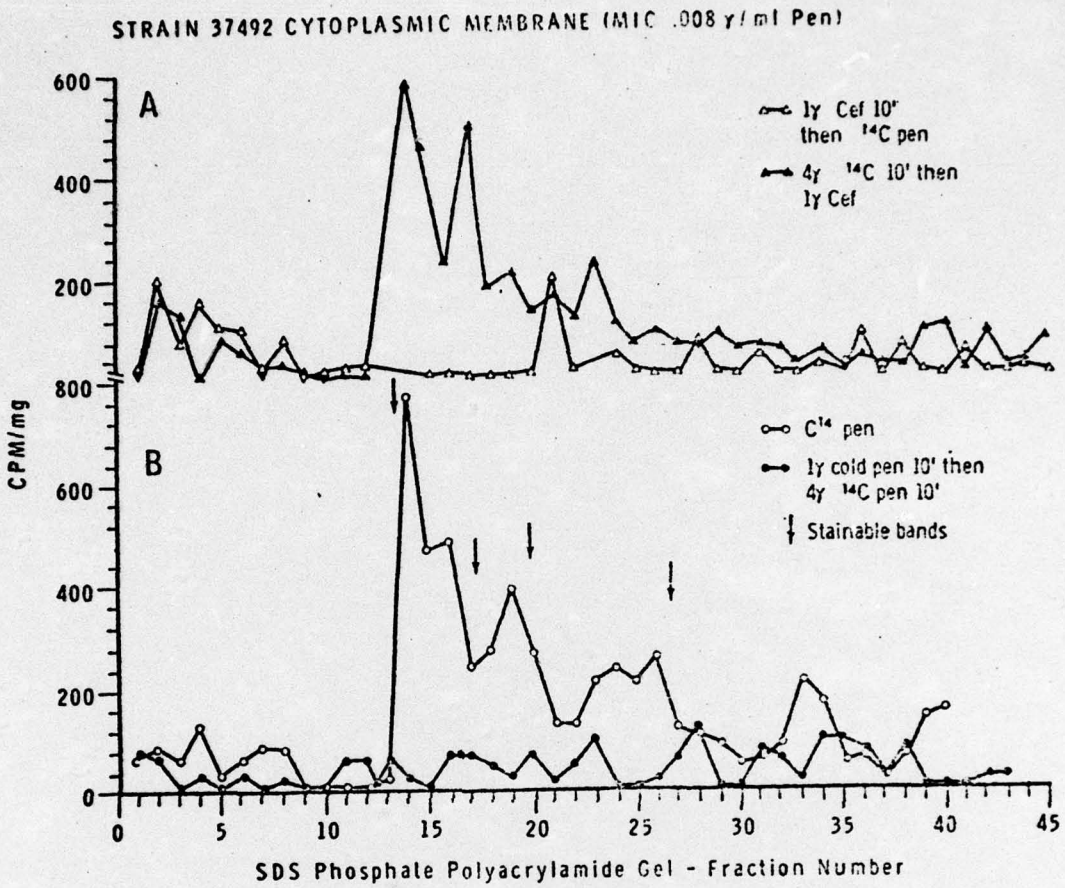


Figure 1

A ▲ - ▲ Membrane from 37492(S) .008γ/ml penicillin exposed to 4γ 14C pen. for 10 minutes, then 1γ cefaloridine for 10 minutes. Reaction stopped as described under materials methods, and subsequently counted. Δ--Δ Same membrane exposed to 1γ cefaloridine for 10' then 14C penicillin.

B O—O Cytoplamic membrane exposed to 14C pen. 4γ/ml-20 minutes
●—● Cytoplamic membrane exposed to 1γ cold penicillin for 10' then 4γ 14C pen. 10'.
The arrow indicate stainable protein bands. (Coomasie Blue)

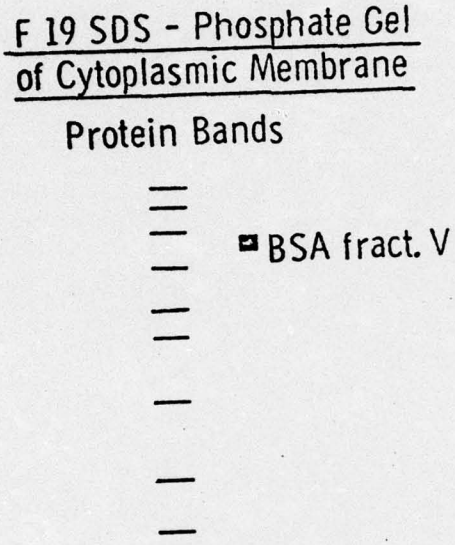


Figure 2

Tracing of F19 stainable bands on SDS polyacrylamide gel electrophoresis. 9 protein bands run in parallel with BSA fract V.

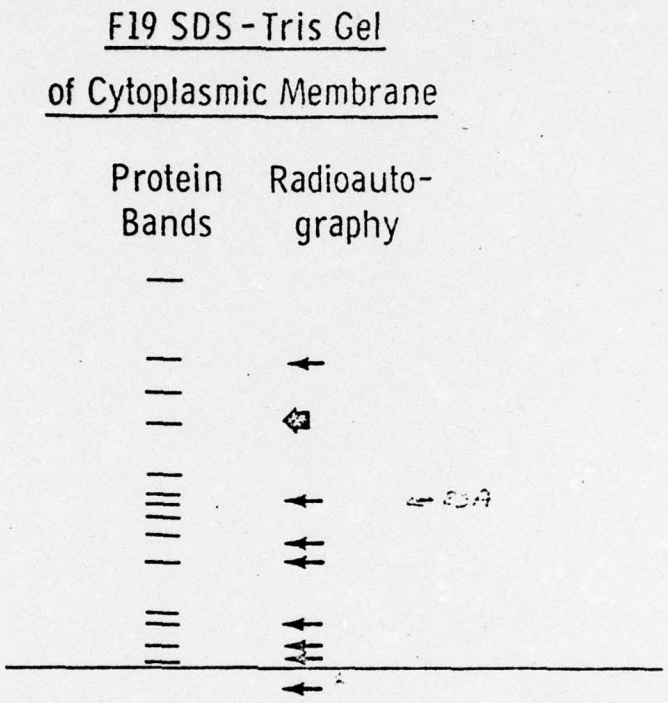


Figure 3

Tracing of F19 SDS-Tris Gel preparation of cytoplasmic membrane; 14 protein bands stain with coomasie blue of these 8* autoradiograph. The thick arrow indicates autoradiograph corresponding to a thick protein band. The continuous horizontal line indicates the position of the tracking dye at the end of the run. *There is one radioactive band migrating faster with no corresponding protein band.

Figure 4

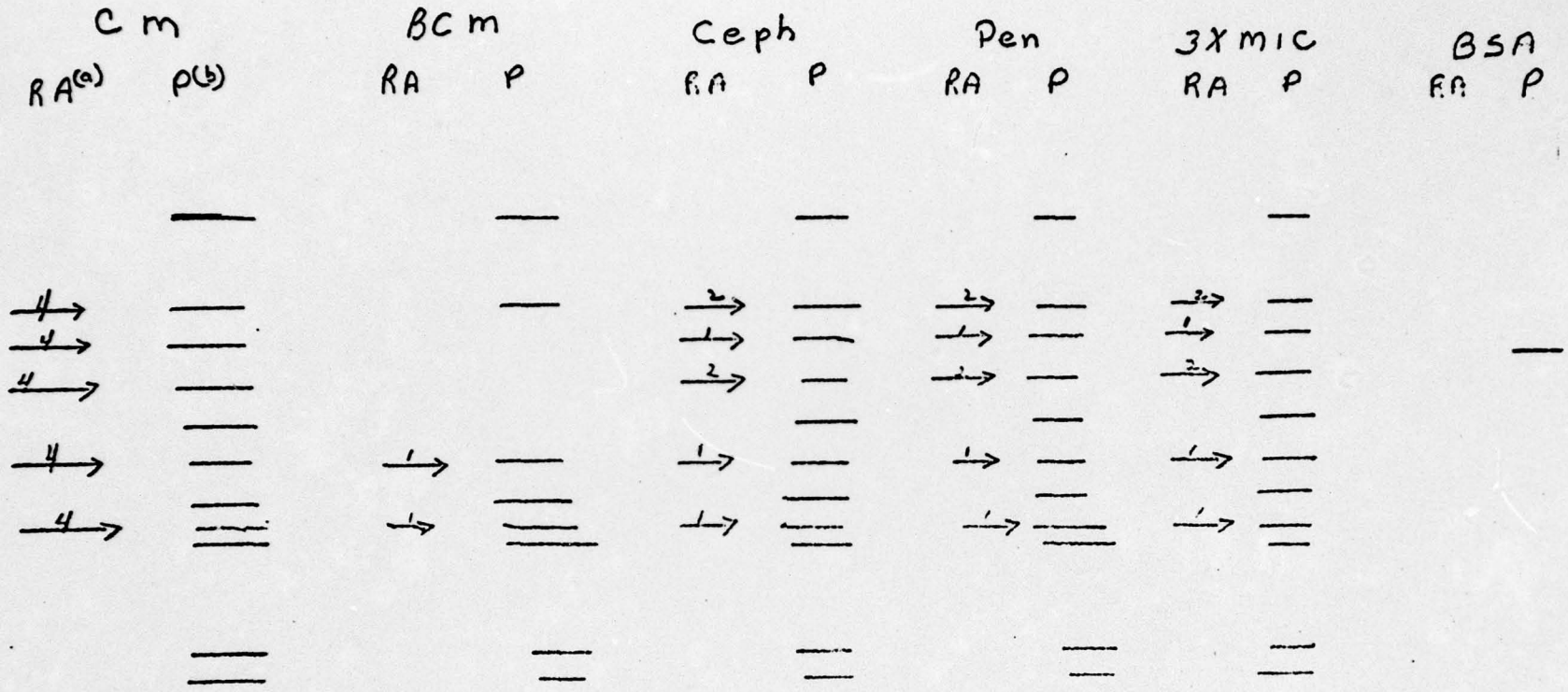
SDS-Tris Gel Electrophoresis of Cytoplasmic Membrane and Autoradiography of Proteins

F18 with an MIC of 0.5 μg/ml was exposed to 20 μg/ml of ^{14}C penicillin with the modifications described under Section C for cytoplasmic membranes.

CM 234 μg protein exposed to 20 μg ^{14}C penicillin.
BCM 328 μg protein exposed to 20 μg ^{14}C penicillin.
Ceph 182 μg protein exposed to 2 μg cephalothin for 10 minutes followed by 20 μg ^{14}Cpenicillin for 10 minutes.
Pen 172 μg of protein exposed to 2 μg of cold penicillin for 10 minutes followed by 20 μg ^{14}C penicillin for 10 minutes.
3xMIC 196 μg of protein exposed to 1.5 μg^{14}C penicillin.
BSA = Bovine Albumin Fraction V.

Horizontal lines mean protein bands obtained after electrophoresis as described under E3. An arrow immediately to the left of a band indicates an autoradiographic protein. The numbers indicate relative intensity (1 lowest, 4 highest) of the radioactive band. Autoradiography plates exposed for 30 days.

FIGURE 4



a : Radio autography

b : Protein Bands

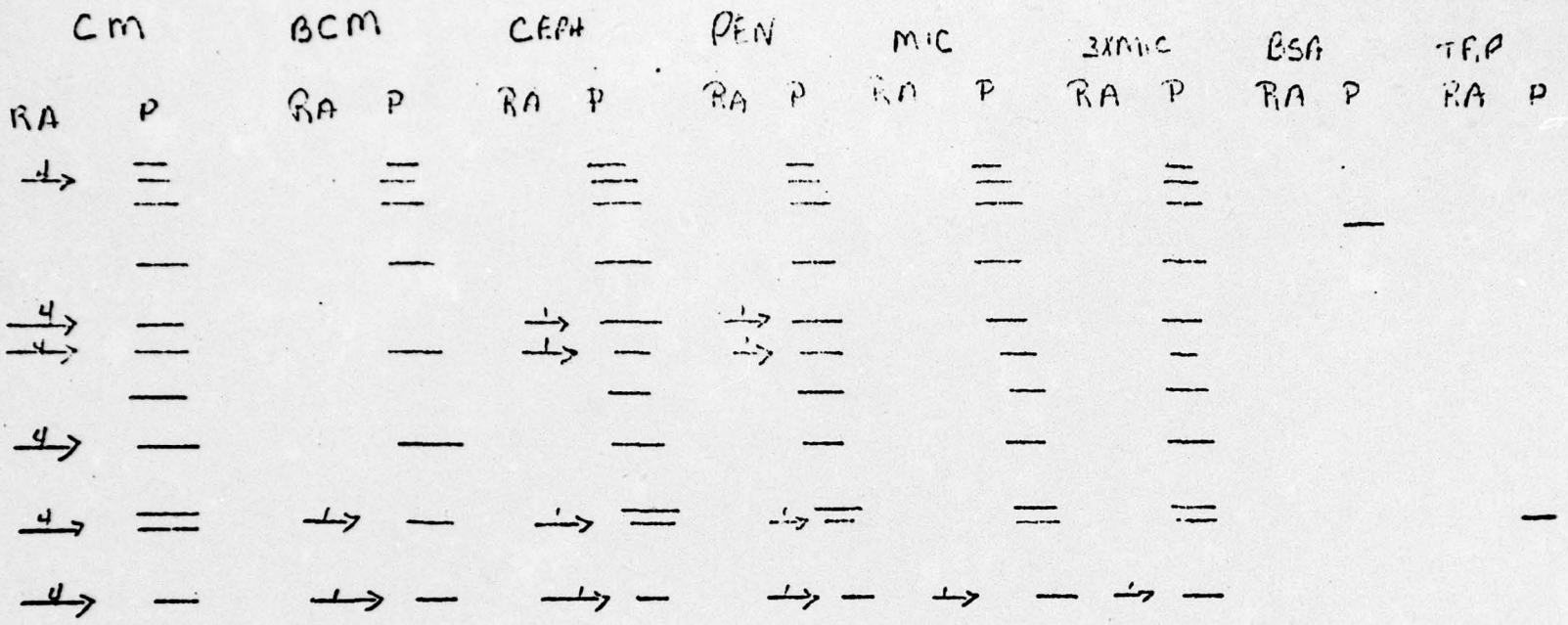


Figure 5 SDS-Tris Gel Electrophoresis of Cytoplasmic Membranes and Autoradiography of Proteins. Strain 33684, MIC 1 μg/ml.

Abbreviations (Numbers in parenthesis are μg of protein treated in preparation.)
Trp = trypsin; CM = cytoplasmic membrane (186 μg); BCM = boiled cell membrane (215 μg); Ceph = Cephaloridine (197 μg); Pen = penicillin (165 μg); MIC - Minimal Inhibitory Concentration (94 μg); 3 times MIC (94 μg).

Autoradiography - 2 months exposure. Arrows and numbers as in Figure 4.

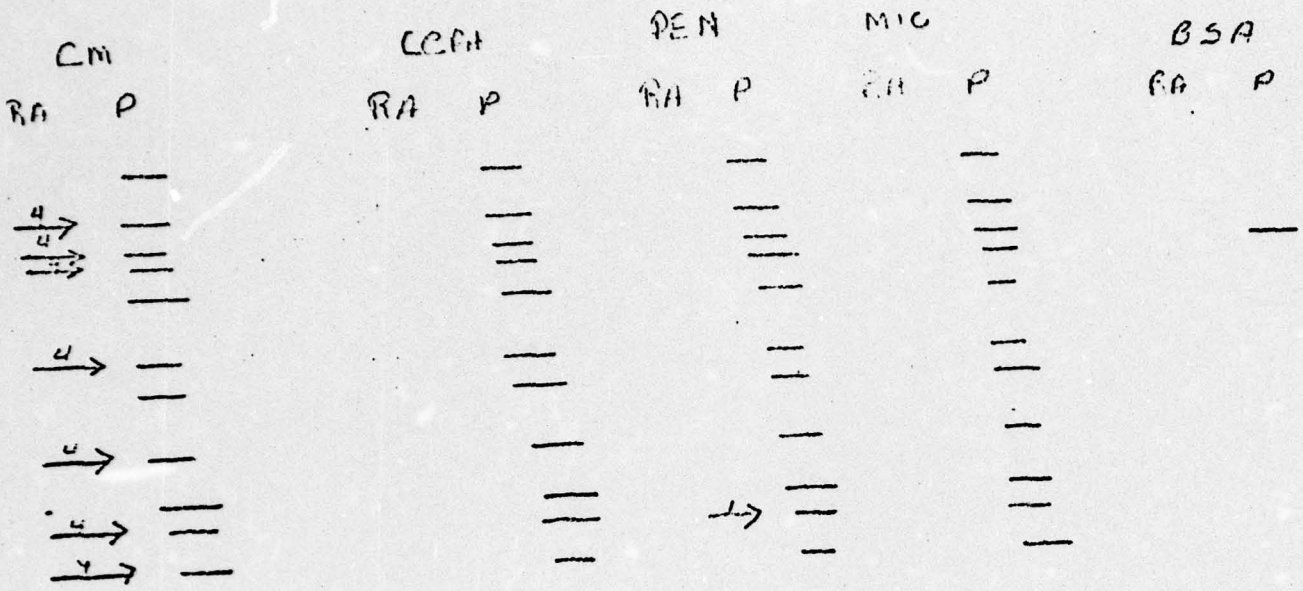


Figure 6 SDS-Tris Gel Electrophoresis of Cytoplasmic Membranes and Autoradiography of Proteins - Strain 1655 MIC 0.05 µg/ml.

Legends, abbreviations, arrows and numbers as in Figure 4.

Amount protein: CM 142 µg, Ceph 146 µg, Cold Penicillin 128 µg, MIC 144 µg.

Autoradiograph plates exposed for 6 weeks.

SUMMARY DADA 17-73-C-3103

It was found during the contract term that an inverse relationship existed between the amount of ^{14}C-penicillin G bound and the sensitivity of Neisseria gonorrhoeae to the antibiotic, i.e., sensitive organisms bound more benzyl penicillin than did resistant organisms. Fractionation of the whole cells indicated that binding occurred at the level of the cytoplasmic membrane and again, the inverse relationship occurred. Specificity of binding was shown by pretreatment of preparations with either unlabeled penicillin or cephalothin, in which instances binding was either strongly inhibited or abolished. From 4-9 different proteins as determined by gel electrophoresis and/or column chromatography bound proteins.

In sum, resistance to penicillin of the gonococcus can be explained by change(s) in the binding proteins of sensitive cells when compared with resistant cells. When the contract ended, we were just starting to characterize the binding proteins.